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Caspase-12 cleavage and increased oxidative stress during motoneuron degeneration in transgenic mouse model of ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by loss of motoneurons in the spinal cord and brain stem. We have characterized motoneuron death in transgenic mice carrying the mutant human copper/zinc superoxide dismutase, as a model for familial ALS. Previous studies have shown the involvement of mitochondria in nerve cell demise in these animals. We report here an early cleavage of caspase-12, residing in the endoplasmic reticulum (ER), in the spinal cord during the course of the disease. Apart from caspase-12, caspase-9, and caspase-3 were activated in the transgenic ALS mice. Staining with an antibody for nitrotyrosine, as a marker for oxidative stress, showed a large increase in the ALS mice. The results indicate that oxidative and ER induced stress causing caspase-12 activation are involved in neuronal death and disease progression in ALS. Caspase-12 and the ER pathway for cell death may constitute potential novel targets for the treatment of ALS.

Keywords: ALS; Caspase-12; Cell death; Endoplasmic reticulum; Oxidative stress; Nitrotyrosine; SOD1

Amyotrophic lateral sclerosis (ALS) is a devastating paralytic disease with the preferential affliction of motoneurons in the ventral spinal cord [1,2]. The underlying cause of the disease is unknown and there is currently no rational treatment or therapy. In 90–95% of patients there is no apparent genetic link (sporadic ALS), in the remaining 5–10% of cases, ALS is inherited in a dominant manner, familial ALS (FALS). Approximately one-fifth of the latter show mutations in Cu/Zn superoxide dismutase 1 (SOD1; EC 1.15.1.1), which is involved in the regulation of oxidative stress, but may also in FALS acquire additional toxic functions [1,2].

Available data indicate that the spinal cord motoneurons in ALS show characteristics of apoptosis with the activation of the cell death machinery and different caspases, causing cell demise [1,2]. Thus both upstream caspases, such as caspase-1, as well as effector caspases, such as caspase-3 and caspase-9 are reported to be activated in the ALS spinal cord [3–5]. In addition, a decrease in the anti-apoptotic protein Bcl-2 is shown to occur in spinal cord of ALS patients [6,7], and the overexpression of Bcl-2 slows disease progress [8], in the transgenic mouse model for ALS (TG-ALS), expressing the mutant human SOD1 gene [9]. Bcl-2 family proteins act at the level of mitochondria, which regulate cell death by controlling the release of cytochrome *c* and other pro-apoptotic molecules [10,11]. Recent reports using minocycline in the TG-ALS model showed a delay of ALS in these mice in conjunction with the inhibition of cytochrome *c* release from the mitochondria [12]. This shows that mitochondrial dysfunction with subsequent alterations in intracellular metabolism are contributing factors to death of motoneurons in ALS.

Apart from the mitochondrial pathway, less is known about the role of other organelles and their dysfunction

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in ALS. In this study, we have analysed the involvement of ER stress and the activation of caspase-12, residing in this organelle [13,14], during the course of the disease in the TG-ALS model. The results showed cleavage of caspase-12 in the spinal cord of the TG-ALS mice prior to the debate of overt symptoms of the disease. Apart from caspase-12, other markers for ER stress, such as the chaperone BiP/Grp78 (BiP), and the transcription factor, CHOP/GADD-153 (CHOP) [15,16], were not altered in the TG-ALS animals. This suggests that in ALS caspase-12 is specifically upregulated and cleaved during the progression of the disease. There was also an increase in nitrotyrosine labelling in the spinal cord motoneurons of TG-ALS mice. Enhanced oxidative stress as shown by increased levels of nitrotyrosine may constitute an upstream factor that can influence and activate caspase-12 in ALS.

Materials and methods

Animals. Transgenic mice expressing mutant human SOD1, with mutation of glycine at position 93 to alanine, $SOD1^{G93A}$, were used as a model for FALS (TG-ALS mice) [9]. The mice were obtained from Jackson Lab, Bar Harbor, Maine, USA, and developed symptoms within six months. As controls, transgenic mice expressing human SOD1 without mutation (SOD2Gur) were used. The mice were sacrificed around day 150 in the group exhibiting no symptoms, or shortly after the debute of the symptoms with paralysis of the hindlimbs in the affected group. A total of about 50 mice were analysed in each group. Approval for animal experiments was obtained from the Local Ethical Committee.

Western blotting. Spinal cord from control, and TG-ALS mice, with or without symptoms was homogenized in ice-cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris–HCl, and 0.1% SDS, pH 8.0) supplemented with the protease inhibitor cocktail (Roche, Germany) [17]. Protein concentrations were determined by protein assay DC (Bio-Rad, Hercules, CA) and equal amounts of protein (20 μg) were separated on a 12% SDS–PAGE by gel electrophoresis. The proteins were transferred to a nitrocellulose membrane (Serva, Heidelberg, Germany), blocked for 1 h in the solution (Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween, 20, and 5% skim milk), and incubated overnight with primary antibody against caspase-12 (diluted 1:500, Oncogene, Boston, MA), caspase-7 (1:1000, Cell Signaling), α-spectrin (1:1000 Chemicon), BiP/GRP78 (1:250, BD Biosciences), CHOP/GADD153 (1:1000, Santa Cruz Biotechnology), β-actin (1:2000, Sigma) or myosin (1:1000: Sigma). After washing, the membrane was incubated with horseradish peroxidase conjugated secondary antibody (1:2000, Jackson ImmunoResearch Laboratories) followed by detection with the enhanced chemiluminescent (ECL) method. Filters were stripped for 30 min at 40 °C (62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 0.68% v/v β-mercaptoethanol) and reprobed.

Immunohistochemistry. Control and TG-ALS mice were anesthetized with 0.5 ml of 5% Avertin and perfused with 10 ml of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The spinal cord was dissected and embedded in paraffin. Five micrometer paraffin sections were deparaffinized and rehydrated in a descending series of ethanol. Antigen retrieval was done in 10 mM citrate buffer (pH 6.0) using a microwave oven and high temperature. Sections were blocked in 1% bovine serum albumin (Sigma) in PBS, or in normal serum, and incubated with primary antibodies against BiP (diluted 1:300) or nitrotyrosine (1:200, Molecular Probes). After washing the sections were incubated with appropriate fluorescent Cy-3 conjugated secondary antibodies (1:200, Jackson Laboratories) and counterstained using 4 μg/ml Hoechst 33342 (Sigma). Sections were analysed using a Leica Axiovert fluorescent microscope.

Real-time PCR. Total RNA was extracted from frozen spinal cord of control and TG-ALS mice, and cDNA was synthesized using dTT primers according to the manufacturer's manual (ThermoScript RT-PCR system, Invitrogen). Real time PCR was performed using the SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK) essentially as described recently [18]. Duplicate cDNA samples were analysed using the following primers:

For caspase-12: forward, 5′-CTCTAACTGTCGGAGTCTGAGAAACA-3′; reverse, 5′-TCAGCAGTGGATATCCCTTTG-3′.
For BiP: forward, 5′-AAGGTGAACGACCCCTAACAAA-3′; reverse, 5′-GTCACTCGGAGAATACCATTAACATCT-3′.
For CHOP: forward, 5′-GTCCCTAGCTTGGCTGACAGA-3′; reverse, 5′-TGGAGAGCGAGGGCTTTG-3′.
For β-actin; forward primer, 5′-CTTCAACACCCCAGCCATG-3′; reverse, 5′-GTGGTACGACCAGAGGCATACA-3′.

Results with real time PCR were normalized against those for β-actin and analysed using Student's t test, ($n = 3-6$). A value of $P < 0.05$ was considered statistically significant.

Results

To study causative factors involved in motoneuron degeneration, we analysed the expression levels and activities of different caspases in control and TG-ALS mice at different stages of the disease. Focusing on upstream caspases, we observed that caspase-12, residing normally within the ER, became cleaved during the early course of the disease (Fig. 1A). Activation of caspase-12 was shown by the appearance of the cleaved fragment, and this was in turn accompanied by a reduction in procaspase-12 levels (Fig. 1A). To study gene expression of caspase-12, we used quantitative PCR as described in Materials and methods. The results showed that the steady state levels of caspase-12 mRNA were significantly upregulated in TG-ALS animals, particularly in animals with overt symptoms (Fig. 1B). These data showing elevated caspase-12 transcripts and low protein levels indicate a rapid turnover and processing of caspase-12 in these animals.

To study whether other caspases are activated in the TG-ALS mice, caspase-9, caspase-7 in addition to caspase-3 were analysed. As shown for caspase-12, caspase-9 was activated in the TG-ALS prior to the appearance of disease symptoms (Fig. 2). Similarly, caspase-3 was also activated, as shown by analysing the cleavage pattern of the caspase-3 substrate [19], α-spectrin (Fig. 2). α-Spectrin also revealed that the activity of calpain increased in the spinal cord of ALS-TG animals, albeit only in animals with disease symptoms (Fig. 2). Caspase-7 was also heavily upregulated in the TG-ALS mice (Fig. 2). However, using the antibodies available, we were unable to reveal any low molecular forms of caspase-7 in these animals.

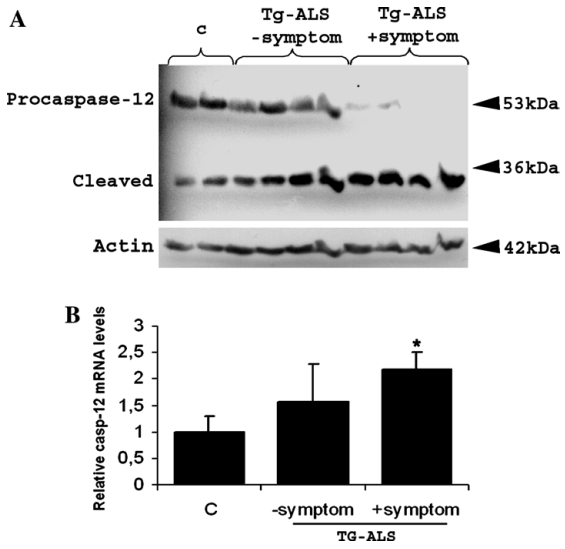

Fig. 1. Cleavage of caspase-12 in spinal cord of TG-ALS mice. (A) Spinal cord lysates were made from control C and TG-ALS mice with or without symptoms. Western blots were made as described in Materials and methods using an antibody specific for caspase-12. β-Actin was used as a control. Note processing of caspase-12 in TG-ALS mice showing no symptoms. (B) Caspase-12 transcripts in mice spinal cord were analysed by quantitative PCR as described in Materials and methods. Note increased caspase-12 levels in TG-ALS mice with symptoms. $^{*}P < 0.05$ for TG-ALS vs. C ($n = 6$).

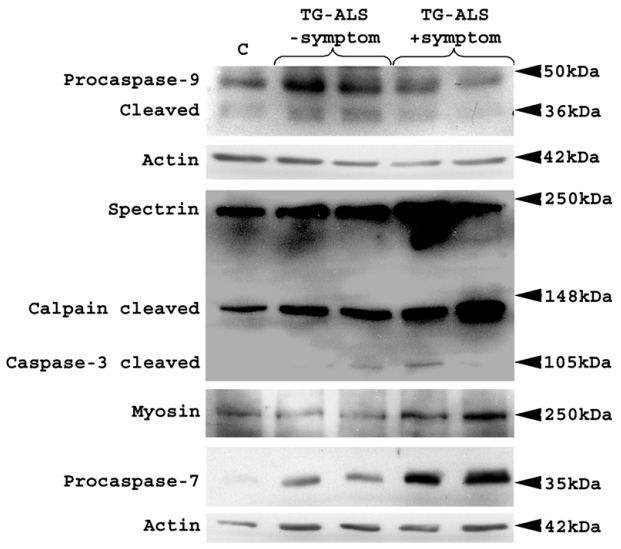

Fig. 2. Cleavage of different caspases and calpain in spinal cord. Spinal cord lysates were made from control C and TG-ALS mice with or without symptoms. Western blots were made using specific antibody for caspase-9, -7, and α-spectrin. β-Actin or myosin were used as controls. Caspase-9. Note the 36 kDa cleavage product of procaspase-9 in TG-ALS mice prior to symtoms. α-Spectrin. The 145 kDa cleaved product is specific for calpain and the 105 kDa one for caspase-3. Note an early activation of caspase-3 in TG-ALS mice and a later increase in calpain activation. Caspase-7. Note increased levels of caspase-7 in TG-ALS mice.

The activation of caspase-12 in different systems has been shown to follow occurrence of ER stress. To study this, we analysed the induction of the ER-chaperone BiP and the transcription factor CHOP that has been associated with changes in gene expression following ER-stress. However, in contrast to caspase-12, we observed no induction of either BiP or CHOP in the TG-ALS animals (Figs. 3A and B). To corroborate data on the protein levels, gene expression was studied using quantitative PCR, which showed no significant changes in BiP or CHOP mRNA in the TG-ALS mice compared with controls (Figs. 3C–D). It should be noted that the changes in these ER proteins may be transient or that they may be expressed by other cells in the spinal cord apart from motoneurons. However, BiP was expressed mainly in the large ventral motoneurons in the spinal cord, as shown in Fig. 4.

To study factors contributing to the processing and induction of caspase-12 in the TG-ALS mice, we studied

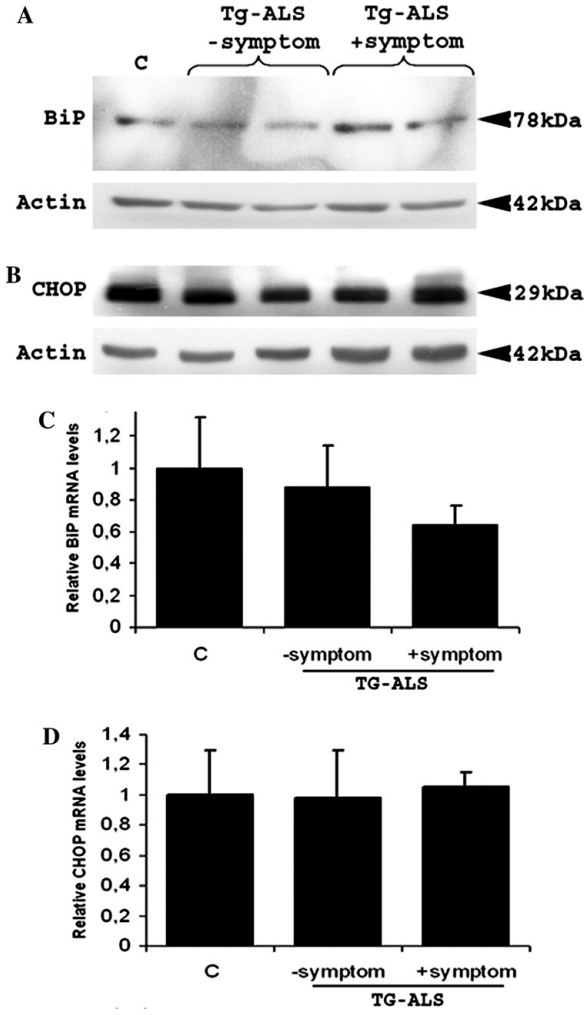

Fig. 3. Expression levels of BiP and CHOP in mice spinal cords. Spinal cord lysates were made from control C and TG-ALS mice with or without symptoms. Western blots were made using specific antibodies. β-Actin was used as a control. (A) There was no change in the ER chaperone BiP. (B) No significant change was noted in the transcription factor CHOP. (C–D) mRNA levels were analysed by quantitative PCR as described in Materials and methods. There was no change in BiP or CHOP mRNA expression.

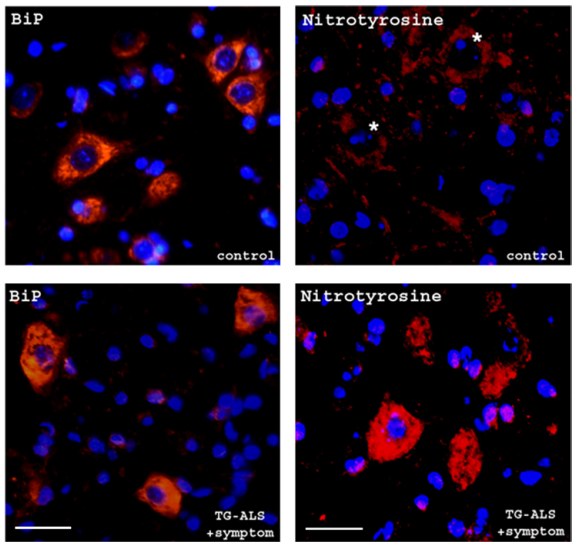

Fig. 4. Immunohistochemistry of ventral spinal cord of control and TG-ALS mice. Immunostaining for BiP and nitrotyrosine (a marker of oxidative stress) in the spinal cord of control and TG-ALS animals was done as described in Materials and methods. Cell nuclei were revealed by Hoechst staining. Scale bar, 10 μm. BiP was present mainly in large cells denoting motorneurons in both control and TG-ALS mice. There is a large increase in nitrotyrosine staining in TG-ALS mice compared with controls. Motoneurons not labelled in controls are shown by asterisk.

the occurrence of oxidative stress in these animals. Fig. 4 shows that there was a large increase in nitrotyrosine labelling, a marker for increased oxidative stress, in the spinal cord of TG-ALS mice.

Discussion

In this study, we have analysed TG-ALS mice carrying the mutant SOD1 gene at different stages of disease progression for changes in expression and activation of caspases, inducing cell death. We compared TG-ALS animals having no obvious signs of the disease with those showing overt symptoms with progressive paralysis of the hindlimbs. Transgenic mice expressing the human wild-type SOD1 gene were used as controls. The results showed that different caspases are altered in the ventral spinal cord from an early stage of the disease. In particular caspase-12, an upstream caspase found normally in an inactive form in the ER [13,14], was readily activated in the spinal cords of the TG-ALS mice, occurring prior to any hindlimb paralysis. As shown by quantitative PCR, caspase-12 mRNA levels were also upregulated in the TG-ALS animals, especially at later stages of the disease. This increase in mRNA expression may be a compensatory phenomenon caused by the rapid turnover and cleavage of caspase-12 in the TG-ALS mice.

Apart from caspase-12, the effector caspases, caspase-9 and caspase-3, were activated in the spinal cord of TG-ALS mice. Caspase-9 is usually involved in the cell death control downstream of mitochondria and is activated in the apoptosome complex [10,11]. However, recent data on the activation pathway for ER induced cell death have shown that caspase-12 may directly or indirectly activate the downstream caspase-9 [20,21]. It was also recently reported using this ALS disease model that caspase-9 is activated in the spinal cord of TG-ALS mice [5]. However, possible upstream caspases or other mechanisms involved in regulation of caspase-9 were not studied further [5]. The present results demonstrate that caspase-12 is cleaved early in the TG-ALS mice probably due to ER related stress, defining an upstream metabolic pathway significant for the pathophysiology of ALS.

Caspase-9 is known to subsequently cleave and activate caspase-3 [10]. Inoue et al. [5] failed to show an increase in caspase-3 in the spinal cord of TG-ALS animals. Studying α-spectrin, a downstream substrate for caspase-3 [19], we observed an increase in activity of caspase-3 in the TG-ALS animals. Caspase-3 cleaves also other cellular proteins, including the inhibitor of the caspase-activated DNase (ICAD), which leads to DNA and chromatin breakdown [10]. In keeping with this there are reports on increased DNA breakage in ALS, as shown by TUNEL labelling of spinal motoneurons in the TG-ALS mice [1,2] as well as in ALS patients [6].

In the present study, caspase-7 was upregulated relatively early in the TG-ALS spinal cords. However, we failed to detect any cleavage of caspase-7 using the available antibodies. The exact role of the activation of caspase-7 in the spinal cord of TG-ALS mice remains thus to be studied in more detail.

Interestingly, we noted that calpain, an enzyme system involved in protein cleavage, was activated in the TG-ALS mice showing clear symptoms. Calpains are activated mainly by elevated intracellular calcium [19] that itself may increase subsequent to changes in different buffering systems in the spinal cord of the TG-ALS with progression of the disease.

To study the extent of ER stress in the TG-ALS spinal cords, we analysed the expression of the ER-specific chaperone BiP and the transcription factor CHOP. BiP has been shown to accompany ER stress and be anti-apoptotic [15], whilst the induction of CHOP is involved in ER stress-induced gene expression mediating cell death [16]. However, we did not observe any significant upregulation of either BiP or CHOP in the spinal cords of TG-ALS mice. This may be due to the fact that BiP and CHOP may increase only transiently in the TG-ALS mice or the proteins may be expressed by other cells than motoneurons. Immunostaining for BiP showed mainly labelling of large motoneurons in the ventral spinal cords of control and TG-ALS mice. Unfortunately, we failed to obtain specific staining of CHOP in the spinal cord using the antibodies available. The data thus suggests that the ER reaction observed in

the TG-ALS mice is specific for caspase-12 and does not directly involve changes in BiP or CHOP as observed in other types of ER-stress [15,16].

ER stress may be part of a general stress response in the cell caused by toxins, misfolded proteins or by changes in calcium and cell metabolism [15,22]. To study the extent of oxidative stress in the TG-ALS animals we analysed the occurrence of protein nitrotyrosinylation in spinal cord sections. The results showed a large increase in staining of nitrotyrosine in spinal cord motoneurons of TG-ALS mice. Increased levels of free 3-nitrotyrosine have been reported before in ALS spinal cords [23] and in TG-ALS mice [24]. Enhanced oxidative stress and peroxide production leading to motoneuron apoptosis have also been observed previously, as studied mainly in cell cultures [25,26]. We report here that the increased levels of nitrotyrosine occurred largely at the same time as the activation of caspase-12 in the TG-ALS mice. The functional significance of increased nitrotyrosination in the progression of ALS together with the elucidation of specific protein substrates affected in the TG-ALS motoneurons remains to be studied further.

Caspase-12, residing in the ER, was earlier found to be involved in death of cultured neuronal cells caused by the Aβ peptide and by misfolded prion protein [13,27]. In the present study, we show that caspase-12 in conjunction with some of the effector caspases is activated in the spinal cords of TG-ALS mice during an early stage of the disease. The processing of caspase-12 may be related to increased oxidative and ER-stress in the spinal cord of the TG-ALS animals.

The human gene homologue of mouse caspase-12 was shown to encode a truncated version of the protein [28], however, activities related to caspase-12 have been identified in different human cells lines and in human pathologies, such as in brains of patients with Creutzfeldt–Jacob disease [27]. The observed activation of caspase-12 in conjunction with ER stress found in the TG-ALS mice may constitute novel targets for designing drugs and future treatment strategies in ALS.

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